REPORT

Study Title

FRESH WATER ALGAL GROWTH INHIBITION TEST WITH

[redacted]

Author

[redacted]

Study completion date

12 August 2008

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

[redacted]

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2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX.

NOTOX B.V.

[redacted]
Study Director

[redacted]

Date: 12 August 2008

[redacted]
Head of In Vitro & Environmental Toxicology

[redacted]

Date: 12/08/2008

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below.
During the on-site process inspections procedures applicable to this type of study were inspected.

The reporting date is the date of reporting to the Study Director. The QAU report was then forwarded to the Test Facility Management.

Type of inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	Protocol	25-Feb-08	25-Feb-08	25-Feb-08
	Report	02-Jul-08	02-Jul-08	02-Jul-08
	Amendment 1 of protocol	12-Aug-08	12-Aug-08	12-Aug-08
Process	**Environmental toxicology**	21-Apr-08	25-Apr-08	29-Apr-08
	Test substance handling			
	Exposure			
	Observations/Measurements			
	Analytical and physical chemistry	11-Feb-08	15-Feb-08	20-Feb-08
	Test substance handling			
	Observations/Measurements			

Head of Quality Assurance
[redacted]

[redacted]

Date: ...12-Aug-08...

4. SUMMARY

Pseudokirchneriella subcapitata, Fresh Water Algal Growth Inhibition Test with [redacted]

The study procedures described in this report were based on the OECD guideline No. 201, 2006. In addition, the procedures were designed to meet the test methods of the EEC directive 92/69, Part C.3, 1992, the ISO International Standard 8692, 2004 and the OECD series on testing and assessment number 23, 2000.

The batch of [redacted] tested was a dark purple powder with a purity of > 95% and not completely soluble in test medium at a loading rate of [redacted]

Based on the results of a range-finding test a final test was performed. Preparation of test solutions started with a loading rate of [redacted] applying a 15-minute treatment period with ultrasonic waves followed by 35 minutes of magnetic stirring to obtain maximum solubility in the test medium. Subsequently, the mixture was left to stabilise for 2 hours after which the Water Soluble Fraction (WSF) was siphoned off. However, very small particles were observed in this WSF. Therefore, the WSF was left to stabilise for another 30 minutes and a second WSF was siphoned off. The lower test concentrations were prepared by subsequent dilutions of the second WSF in test medium. The final test solutions were all clear and ranged from colourless to dark pink.

Exponential growing algal cultures were exposed to a control and to 0.32, 1.0, 3.2, 10, 32 and 100% of the WSF. The initial cell density was 10^4 cells/ml and the total test period was 72 hours. Samples for possible analysis were taken at the start, after 24 hours of exposure and at the end of the test.

The analytical results showed that the method used was not suitable for the two lowest test concentrations. However, these concentrations were not needed for the calculation of the EC_{50} values and could therefore be omitted from further calculations. The other, higher, concentrations showed a relatively high recovery in the samples taken at the end of the test period. The reason for this is not known. Average exposure concentrations of the test substance based on the [redacted], respectively, were calculated. These results showed that the initial test concentrations based on the [redacted] were similar and that these concentrations remained stable during the test period. Test substance concentrations based on the [redacted] were significantly lower at the start of the test and, especially at the lower concentrations, did not remain stable. Taken the worst case scenario in account, effect parameters were based on the average exposure concentrations of [redacted] based on [redacted] i.e. 0.21, 0.86, 5.4 and 23 mg/l.

The study met the acceptability criteria prescribed by the protocol and was considered valid.

[redacted] reduced growth rate of this fresh water algae species significantly at average exposure concentrations of 0.86 mg/l and higher.

The EC_{50} for growth rate reduction (E_RC_{50}: 0-72h) was 12 mg/l with a 95 % confidence interval ranging from 7.7 to 18 mg/l.

The EC_{50} for yield inhibition (E_YC_{50}: 0-72h) was 1.2 mg/l with a 95 % confidence interval ranging from 0.39 to 3.7 mg/l.

The NOEC for growth rate reduction was 0.21 mg/l, while the NOEC for yield inhibition was < 0.21 mg/l.

Effect parameters were based on a worst case scenario. It can not be excluded that at least part of the effect was due to absorption of wavelengths necessary for algal growth by the colour of the test solutions.

5. INTRODUCTION

5.1. Preface

Sponsor	[redacted]
Study Monitor	[redacted]
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	[redacted]
Technical Coordinator	[redacted]
Principal Scientist	[redacted]
Study Plan	Start : 17 March 2008 Completion : 08 May 2008

5.2. Aim of the study

The purpose of the study was to evaluate the test substance for its ability to generate toxic effects in *Pseudokirchneriella subcapitata* during an exposure period of at least 48 and at most 96 hours and, if possible, to determine the EC_{50} for both reduction of growth rate and inhibition of yield.

5.3. Guidelines

The study procedures described in this report were based on the Organization for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, guideline No. 201: "Freshwater Alga and Cyanobacteria, Growth Inhibition Test", Adopted March 23, 2006.

In addition, the procedures were designed to meet the test methods prescribed by the following guidelines and guidance documents:

- European Economic Community (EEC), EEC directive 92/69, Part C: Methods for the determination of ecotoxicity, Publication No. L383, December 1992, C-3: "Algal Inhibition Test".
- ISO International Standard 8692: "Water quality - Freshwater algal growth inhibition test with unicellular green algae", Second edition, 01 October 2004.
- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, December 14, 2000.

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 2 years after finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. NOTOX will retain information concerning decisions made.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

5.5. Definitions

Cell density is the number of cells per millilitre.

Growth rate is the increase in cell density per unit time. It is derived from the slope of the growth curve in a logarithmic plot. Following from the mathematical nature of exponential growth, the measure of the specific growth rate is preferable over biomass or yield. The E_RC_{50} is the concentration of test substance that results in a 50% reduction in growth rate relative to the control.

Yield is defined as the biomass at the end of the exposure period minus the biomass at the start of the exposure period. The E_yC_{50} is the concentration of test substance that results in a 50% inhibition of yield relative to the control.

No Observed Effect Concentration (NOEC) is the highest concentration tested at which the measured parameter(s) show(s) no significant effect on algal growth relative to control values.

6. MATERIALS AND METHODS

6.1. Test Substance

6.1.1. Test substance information

Identification	[redacted]
Structure	[redacted]
Molecular formula	[redacted]
Molecular weight	1317.45
Description	Dark purple powder
Batch	[redacted]
Purity	>95% (NMR)
Test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	01 July 2008

6.1.2. Study specific test substance information

Stability in water	Not indicated
Solubility in water	Poorly

6.1.3. Reference substance

This report includes the results of the most recent reference test with potassium dichromate (Appendix V).

6.1.4. Preliminary data

The water solubility for the [redacted] respectively (NOTOX [redacted] The 48h-EC_{50} of [redacted] for mobility of *Daphnia magna* was 2.8 mg/l based on average measured concentrations of the whole product (NOTOX [redacted]

6.2. Test System

Species	*Pseudokirchneriella subcapitata*, strain: NIVA CHL 1
Source	In-house laboratory culture.
Reason for selection	This system is an unicellular algal species sensitive to toxic substances in the aquatic ecosystem and has been selected as an internationally accepted species.

6.3. Fresh water algae culture

Stock culture	Algae stock cultures were started by inoculating growth medium with algal cells from a pure culture on agar. The suspensions were continuously aerated and exposed to light in a climate room at a temperature of 21-24°C.
Light intensity	60 to 120 µE/m^2/s when measured in the photosynthetically effective wavelength range of 400 to 700 nm.
Stock culture medium	M1; according to the NPR 6505, formulated using Milli-Ro water (tap-water purified by reverse osmosis; Millipore Corp., Bedford, Mass., USA) with the following composition:

$NaNO_3$	500	mg/l
K_2HPO_4	40	mg/l
$MgSO_4.7H_2O$	76	mg/l
$Na_2CO_3.10H_2O$	54	mg/l
$C_6H_8O_7.H_2O$	6	mg/l
NH_4NO_3	330	mg/l
$CaCl_2.H_2O$	36	mg/l
$C_6H_5FeO_7.xH_2O$	6	mg/l
H_3BO_3	2.9	mg/l
$MnCl_2.4H_2O$	1.81	mg/l
$ZnCl_2$	0.11	mg/l
$CuSO_4.5H_2O$	0.08	mg/l
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.018	mg/l

Pre-culture	3 days before the start of the test, cells from the algal stock culture were inoculated in culture medium at a cell density of 1 x 10^4 cells/ml. The pre-culture was maintained under the same conditions as used in the test. The cell density was measured immediately before use.

Pre-culture medium

M2; according to the OECD 201 Guideline, formulated using Milli-Q water (tap water purified by reverse osmosis (milli-RO) and subsequently passed over activated carbon and ion-exchange cartridges: Milli-Q water; Millipore Corp., Bedford, Mass., USA) preventing precipitation and with the following composition:

NH_4Cl	15	mg/l
$MgCl_2.6H_2O$	12	mg/l
$CaCl_2.2H_2O$	18	mg/l
$MgSO_4.7H_2O$	15	mg/l
KH_2PO_4	1.6	mg/l
$FeCl_3.6H_2O$	64	µg/l
$Na_2EDTA.2H_2O$	100	µg/l
H_3BO_3	185	µg/l
$MnCl_2.4H_2O$	415	µg/l
$ZnCl_2$	3	µg/l
$CoCl_2.6H_2O$	1.5	µg/l
$CuCl_2.2H_2O$	0.01	µg/l
$Na_2MoO_4.2H_2O$	7	µg/l
$NaHCO_3$	50	mg/l
Hardness (Ca+Mg)	0.24	mmol/l (24 mg $CaCO_3$/l)
pH	8.1 ± 0.2	

6.4. Preparation of test solutions

The standard test procedures required generation of test solutions, which contained completely dissolved test substance concentrations or stable and homogeneous mixtures or dispersions. The testing of concentrations that would disturb the test system was prevented as much as possible (e.g. film of the test substance on the water surface).

The batch of [redacted] tested was a dark purple powder with a purity of > 95% and not completely soluble in test medium at a loading rate of [redacted]

Preparation of test solutions started with a loading rate of [redacted] applying a 15-minute treatment period with ultrasonic waves followed by 30-35 minutes of magnetic stirring to obtain maximum solubility in the test medium. Subsequently, these mixtures were left to stabilise for 2-2¼ hours after which the Water Soluble Fraction (WSF) was siphoned off. The WSF prepared for the final test was left to stabilise for another 30 minutes and a second WSF was siphoned off because very small particles were observed in the first one. The lower test concentrations were prepared by subsequent dilutions of the WSF in test medium. The final test solutions were all clear and ranged from colourless to dark pink.

After preparation, volumes of 50 ml were added to each replicate of the respective test concentration. Subsequently, 1 ml of an algal suspension was added to each replicate providing a cell density of 10^4 cells/ml.

6.5. Range-finding test

A range-finding test was performed to provide information about the range of concentrations to be used in the final test. Test procedure and conditions were similar to those applied in the final test with the following exceptions:

- Exponentially growing algal cultures were exposed to 0.10, 1.0, 10 and 100% of a WSF prepared at a loading rate of [redacted]
- Three replicates were tested per concentration and three replicates in the control group;
- Algae used to initiate the test were taken directly from the culture;
- pH was only measured in the control and the highest test concentration;
- No sampling for determination of actual test concentrations was performed;
- Volume of the test medium was 50 ml.

6.6. Final test

6.6.1. Test concentrations

[redacted]	0.32, 1.0, 3.2, 10, 32 and 100% of a WSF prepared at a loading rate of [redacted]
Controls	Test medium without test substance or other additives
Replicates	3 replicates of each test concentration 6 replicates of the control In addition, extra replicates with and/or without algae were taken for sampling purposes and background measurements

6.6.2. Test procedures and conditions

Test duration	72 hours
Test type	Static
Test vessels	50 ml Petri dishes, containing 40 ml of test solution
Medium	M2
Cell density	An initial cell density of 1 x 10^4 cells/ml.
Illumination	Continuously using TLD-lamps of the type 'Gro-lux' of 30 Watt, with a light intensity within the range of 110 to 115 $\mu E.m^{-2}.s^{-1}$
Incubation	Vessels were distributed at random in the incubator. During incubation the algal cells were kept in suspension by continuous shaking.

6.6.3. Sampling for analysis of test concentrations

During the final test samples for possible analysis were taken from all test concentrations and the control according to the schedule below. The method of analysis is described in the appended Analytical Report (Appendix VI).

Frequency	at t=0 h, t=24 h and t=72 h
Volume	2 ml
Storage	Samples were stored in a freezer until analysis.

At the end of the exposure period, the replicates with algae were pooled at each concentration before sampling.

Compliance with the Quality criteria regarding maintenance of actual concentrations was demonstrated by running a test vessel at the highest substance concentration but without algae and samples for analysis were taken at the start, after 24 hours of exposure and at the end of the test period.

Additionally, singular reserve samples of 2 ml were taken from all test solutions for possible analysis. If not already used, these samples were stored in a freezer for a maximum of three months after delivery of the draft report, pending on the decision of the sponsor for additional analysis.

6.6.4. Measurements

pH	At the beginning and at the end of the test. The pH of the solutions should preferably not deviate by more than 1.5 units during the test.
Temperature of medium	Continuously in a temperature control vessel.

6.6.5. Recording of cell densities

At the beginning of the test, cells were counted using a microscope and a counting chamber. Thereafter cell densities were determined by spectrophotometric measurement of samples at [redacted] using a Varian Cary 50 single beam spectrophotometer with immersion probe ([redacted] Algal medium was used as blank and one extra test vessel per concentration without algae was used as background for the determination of the algal cell density at each time interval. One exception was made for the 1.0% WSF at the last day of the final test. Algae exposed to this concentration were counted using a microscope and a counting chamber because the accompanying vessel without algae was lost for use as background.

6.7. Electronic data capture

Observations/measurements in the study were recorded electronically using the following programme(s):
Cary 50 single beam spectrophotometer including Cary UVVIS Pharma Upgrade Version 3.1 software (Varian, Mulgrave, Australia): Algal cell density.
REES version 1.5 (REES scientific, Trenton, NJ, USA): Temperature.

6.8. Interpretation

6.8.1. Data handling

Calibration curve
Quantification of cell densities was based on a calibration curve. Cell density was plotted versus extinction using spectrophotometric measurements of a minimum of six dilutions of an algal suspension with different cell densities. The calibration curve was composed using linear regression. The software automatically calculates the cell densities based on this curve for the spectrophotometric measurements at the various points in time during the test period.

Comparison of average growth rates
The average specific growth rate for a specific period is calculated as the logarithmic increase in the biomass from the equation for each single vessel of controls and treatments:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} (\text{day}^{-1})$$

Where: μ_{i-j} = the average specific growth rate from time i to j
X_i = the biomass at time i
X_j = the biomass at time j

The average growth rate at each test substance concentration is then compared with the control value and the percentage reduction in growth rate is calculated:

$$\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100$$

Where: $\%I_r$ = percent inhibition in average specific growth rate
μ_C = mean value for average specific growth rate in the control group
μ_T = average specific growth rate for the treatment replicate

Yield
The percent inhibition in yield is calculated for each treatment replicate as follows:

$$\%I_y = \frac{Y_C - Y_T}{Y_C} \times 100$$

Where: $\%I_y$ = percent inhibition of yield
Y_C = mean value for yield in the control group
Y_T = value for yield for the treatment replicate

Growth inhibition is calculated for the total period of exposure.

Determination of the average exposure concentrations
The average exposure concentrations were calculated as the Time Weight Average (TWA) of the concentrations of [redacted] measured in the samples taken at the start ($C_{t=0}$), after 24 hours ($C_{t=24}$) and the end of the test ($C_{t=72}$):

$$\frac{24 \times \sqrt{C_{t=0} \times C_{t=24}} + 48 \times \sqrt{C_{t=24} \times C_{t=72}}}{72}.$$

In case concentrations measured were below the limit of detection, the final exposure concentration(s) were taken as a factor of 2 below this limit. This procedure is based on the OECD "Guidance document on the use of the harmonised system for the classification of chemicals which are hazardous for the aquatic environment".

Determination of the NOEC and calculation of the EC_{50}
For determination of the NOEC and the EC_{50} the approaches recommended in the OECD guideline 201 were used. An effect was considered to be significant if statistical analysis of the data obtained for the test concentrations compared with those obtained in the negative control revealed significant reduction of growth rate or inhibition of yield (ANOVA, William's Test, TOXSTAT Release 3.5, 1996, D.D. Gulley, A.M. Boelter, H.L. Bergman). Additionally, the EC_{10} was determined to meet the recommendations as put down in "A Review of Statistical Data Analysis and Experimental Design in OECD Aquatic Toxicology Test Guidelines" by S. Pack, August 1993. Calculation of the EC_{50} and EC_{10} values was based on log-linear regression analysis of the percentages of growth rate reduction and the percentages of yield inhibition versus the logarithms of the corresponding average exposure concentrations of the test substance based on the [redacted]

6.8.2. Acceptability of the test

1. In the controls, cell density increased by an average factor of > 16 within 2 days.
2. The mean coefficient of variation for section-by-section specific growth rates in the control cultures did not exceed 35%.
3. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures did not exceed 7%.

6.9. List of deviations

6.9.1. List of protocol deviations

1. Algae used for the range-finding test originated from the stock culture instead of a pre-culture that was incubated for 2-4 days prior to study start.
   Evaluation: Growth in the pre-culture was not sufficient. Consequently, algae from the stock culture were used.
2. Light intensity during the range finding test was within 112 and 123 $\mu E.m^{-2}.s^{-1}$.
   Evaluation: A slight exceeding of the optimum range does not affect algal growth.
3. Variation on section-by section growth rate in the control group was above 35% at the end of the range-finding test (i.e. 45%).

Evaluation: The aim of the range-finding test, i.e. to provide information about the range of concentrations to be used in the final test, was not affected by this.

4. Both the range-finding and the final test were performed in Petri-dishes.
   Evaluation: In order to reduce the light path by reducing the depth of the coloured solutions.
5. Appearance of the algal cells was not checked at the end of the final test.
   Evaluation: This endpoint is only optional and not obligatory according to the OECD Guideline 201.

The study integrity was not adversely affected by the deviations.

6.9.2. List of standard operating procedures deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

7. RESULTS

7.1. Range-finding test

7.1.1. Mean cell densities, reduction of growth rate and inhibition of yield

The mean cell densities measured during the range-finding test are presented in Table 1. Table 2 presents the percentages growth rate reduction and yield inhibition per concentration. Reduction of growth rate and inhibition of yield were observed in the solutions containing 1.0, 10 and 100% of the WSF prepared at a loading rate of [redacted] The expected EC_{50} for growth rate reduction was between concentrations present in 10 and 100% of the WSF. The expected EC_{50} for yield inhibition was between concentrations present in 0.10 and 1.0% of the WSF.

Note that the concentrations, in which effects were observed, were all coloured. Colour ranged from very slightly pink at the 1.0% WSF to dark pink at the 100% WSF. It can therefore not be excluded that at least part of the effect was due to absorption of wavelengths necessary for algal growth by the colour of the test solutions. However, it was not expected that the EC_{50} for algal growth rate reduction would be below the EC_{50} for immobilization of *Daphnia magna*. Therefore, the study was continued with a final test, under similar conditions as in the range-finding test.
All test conditions were maintained within the limits prescribed by the protocol.

Table 1 Mean cell densities (x10^4 cells/ml) during the range-finding test

[redacted] % WSF prep. at [redacted]	Exposure time (hours)			
	0	24	48	72
control	1.0	2.8	12.6	164.9
0.10	1.0	4.3	23.7	161.8
1.0	1.0	4.7	17.9	60.9
10	1.0	4.4	16.0	40.5
100	1.0	1.5	3.6	2.7

Table 2 Percentage reduction of growth rate and inhibition of yield during the range-finding test

[redacted] % WSF prep. at [redacted]	Mean growth rate		Yield (0-72 h)	
	μ (0-72 h)	Reduction (%)	$x10^4$ cells/ml	Inhibition (%)
control	0.07090		163.95	
0.10	0.07043	0.7	160.81	1.9
1.0	0.05704	19.5	59.93	63.4
10	0.05138	27.5	39.54	75.9
100	0.01369	80.7	1.71	99.0

7.2. Final test

7.2.1. Measured test substance concentrations

The results of analysis of the samples taken during the final test are described in Tables 4, 5 and 6 of the appended Analytical Report.

The analytical results showed that the method used was not suitable for the two lowest test concentrations. However, these concentrations were not needed for the calculation of the EC_{50} values and could therefore be omitted from further calculations. The other, higher, concentrations showed a relatively high recovery in the samples taken at the end of the test period. The reason for this is not known. Table 3 gives the average exposure concentrations of the test substance based on the [redacted], respectively. These results showed that the initial test concentrations based on the [redacted] were similar and that these concentrations remained stable during the test period. Test substance concentrations based on the [redacted] were significantly lower at the start of the test and, especially at the lower concentrations, did not remain stable. Taken the worst case scenario in account, effect parameters were based on the average exposure concentrations of [redacted] based on [redacted]

Table 3 Measured concentrations versus nominal concentrations

[redacted]	[redacted] % WSF	Concentration of [redacted] (mg/l)				
		t=0h	t=24h	t=72h	Average	% of initial
	3.2	2.55	2.57	4.25	3.1	120
	10	8.31	7.80	8.77	8.2	99
	32	25.0	25.2	45.7	31	124
	100	83.3	98.8	123	104	124
	3.2	2.17	1.98	3.03	2.3	107
	10	6.86	6.90	6.91	6.9	101
	32	21.5	22.7	33.7	26	120
	100	69.9	88.2	94.0	87	124
	3.2	0.819	<LOD	<LOD	0.21	25
	10	2.22	0.569	0.915	0.86	39
	32	6.83	4.54	6.21	5.4	79
	100	25.3	22.4	24.2	23	93

7.2.2. Mean cell densities

Table 4 shows mean cell densities measured at 24-hour intervals at the different concentrations of [redacted] The respective growth curves are shown in Figure 1 (see Appendix I for the cell densities per replicate).

Table 4 Mean cell densities (x 10^4 cells/ml) during the final test

Average conc. [redacted] (mg/l)	Exposure time (hours)			
	0	24	48	72
control	1.0	5.9	69.6	328.5
< LOD	1.0	5.6	52.6	307.3
< LOD	1.0	5.3	50.2	292.8
0.21	1.0	5.9	41.3	270.5
0.86	1.0	6.6	44.5	223.1
5.4	1.0	5.5	15.7	42.0
23	1.0	1.0	7.1	9.3

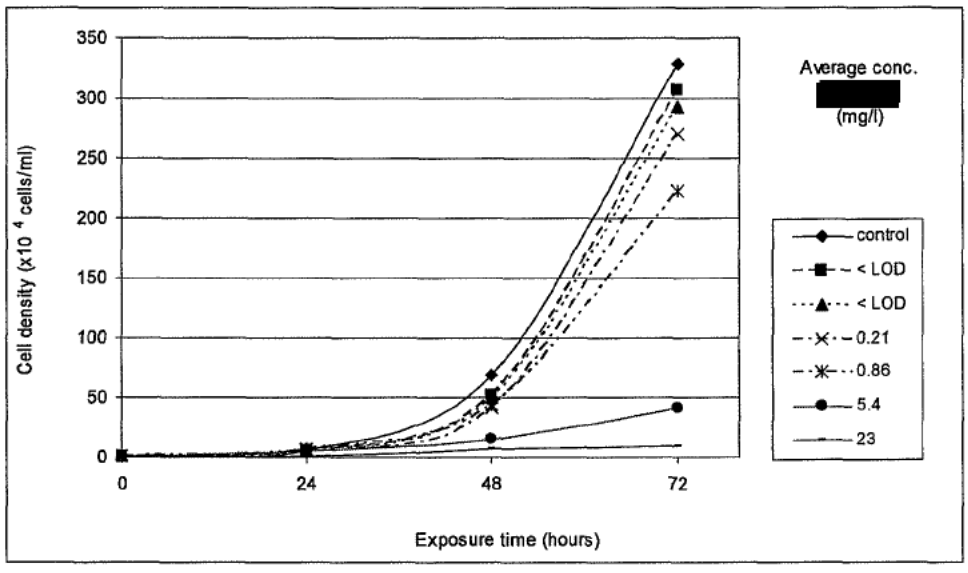

Figure 1 Growth curves at different concentrations of [redacted]

7.2.3. Reduction of growth rate and inhibition of yield

Table 5 shows the calculation of the percentages of growth rate reduction (total test period) and the percentages of yield inhibition. Table 6 shows the calculation of the percentages of growth rate reduction at different time intervals (see Appendix I for the values of growth rate and yield per replicate). Statistical analysis of the data is shown in Appendices II and III.

Growth rates were in the range of the controls at average concentrations up to and including 0.21 mg/l during the 72-hour test period, whereas the growth rate of algae exposed to 0.86 mg/l and higher were increasingly reduced. Reduction of growth rate increased during the first 48 hours of exposure and decreased during the last 24 hours of exposure, which may be related to a decrease of the [redacted]t 2. This increase followed by decrease pattern did not apply to the highest test concentration. However, effects on growth at the highest concentration were most probably intensified by the colour of the test substance.
Statistically significant reduction of growth rate was found at test concentrations of 0.86 mg/l and higher (William's Test, $\alpha = 0.05$).

Inhibition of yield increased with increasing concentration of [redacted] from the lowest concentration tested upwards resulting in 98% inhibition at 23 mg/l. Statistically significant inhibition of yield was found at test concentrations of 0.86 mg/l and higher (William's Test, $\alpha = 0.05$). However, the two concentrations directly below 0.86 mg/l should be considered as biologically significantly inhibited (i.e. inhibition > 10%).

Table 5 Percentage reduction of growth rate (total test period) and percentage inhibition of yield during the final test

Average conc. [redacted] (mg/l)	Mean growth rate		Yield (0-72 h)	
	μ (0-72 h)	Reduction (%)	$x10^4$ cells/ml	Inhibition (%)
control	0.08041		327.48	
< LOD	0.07954	1.1	306.30	6.5
< LOD	0.07744	3.7	291.75	10.9
0.21	0.07778	3.3	269.53	17.7
0.86	0.07503	6.7	222.09	32.2
5.4	0.05185	35.5	40.97	87.5
23	0.03078	61.7	8.33	97.5

Table 6 Percentage reduction of growth rate at different time intervals during the final test

Average conc. [redacted] (mg/l)	Mean growth rate					
	μ (0-24 h)	Reduction (%)	μ (24-48 h)	Reduction (%)	μ (48-72 h)	Reduction (%)
control	0.07293		0.10264		0.06565	
< LOD	0.07165	1.8	0.09334	9.1	0.07362	-12.1
< LOD	0.06848	6.1	0.09415	8.3	0.06968	-6.1
0.21	0.07420	-1.7	0.08073	21.3	0.07840	-19.4
0.86	0.07837	-7.5	0.07943	22.6	0.06729	-2.5
5.4	0.07085	2.8	0.04385	57.3	0.04083	37.8
23	0.00000	100.0	0.07848	23.5	0.02043	68.9

7.2.4. Determination of effect concentrations

Table 7 shows the effect parameters based on average exposure concentrations, see also Appendix IV.

Table 7 Effect parameters

Parameter	Concentration [redacted] (mg/l)	95%-confidence interval
NOE_RC	0.21	
72h-E_RC_{10}	1.1	0.69 - 1.7
72h-E_RC_{50}	12	7.7 - 18
NOE_YC	< 0.21	
72h-E_YC_{10}	0.19	0.06 - 0.64
72h-E_YC_{50}	1.2	0.39 - 3.7

7.2.5. Experimental conditions

Table 8 shows the pH recorded at the beginning and the end of the test. The pH was within the limits prescribed by the protocol (6.0-9.0, preferably not varying by more than 1.5 unit). During the exposure period the temperature measured in the incubator was maintained between 21.9 and 23.1°C. Temperature remained within the limits prescribed by the protocol (21-24°C, constant within 2°C).

Table 8 pH levels recorded during the final test

Average conc. [redacted] (mg/l)	Exposure time (hours)	
	0	72
control	8.1	7.9
< LOD	8.2	8.0
< LOD	8.3	8.0
0.21	8.3	8.0
0.86	8.2	8.1
5.4	8.2	8.0
23	8.2	8.1

8. CONCLUSION

Under the conditions of the present study with *Pseudokirchneriella subcapitata*, [redacted] reduced growth rate of this fresh water algae species significantly at average exposure concentrations of 0.86 mg/l and higher.

The EC_{50} for growth rate reduction (E_RC_{50}: 0-72h) was 12 mg/l with a 95 % confidence interval ranging from 7.7 to 18 mg/l.

The EC_{50} for yield inhibition (E_YC_{50}: 0-72h) was 1.2 mg/l with a 95 % confidence interval ranging from 0.39 to 3.7 mg/l.

The NOEC for growth rate reduction was 0.21 mg/l, while the NOEC for yield inhibition was < 0.21 mg/l.

Effect parameters were based on a worst case scenario. It can not be excluded that at least part of the effect was due to absorption of wavelengths necessary for algal growth by the colour of the test solutions.

APPENDIX I WORKSHEET DATA

Table 9 Individual cell densities

Number of inoculated cells at t=0:		1 $x10^4$ cells/ml			
Average conc. [redacted] mg/l	Vessel number	Exposure time (hours)			
		0	24	48	72
control	1	1.00	8.25	56.95	314.52
	2	1.00	5.29	44.32	387.83
	3	1.00	4.96	63.34	280.45
	4	1.00	5.24	79.97	306.31
	5	1.00	6.23	84.76	340.77
	6	1.00	5.15	88.09	340.98
< LOD	1	1.00	5.11	46.59	285.93
	2	1.00	5.57	53.74	321.43
	3	1.00	6.11	57.59	314.54
< LOD	1	1.00	5.16	46.81	270.00
	2	1.00	6.56	61.74	460.50
	3	1.00	4.09	42.12	147.75
0.21	1	1.00	6.43	38.78	281.38
	2	1.00	5.90	44.63	269.13
	3	1.00	5.51	40.39	261.09
0.86	1	1.00	5.70	37.60	190.59
	2	1.00	6.78	44.98	240.91
	3	1.00	7.30	50.79	237.78
5.4	1	1.00	5.50	15.54	47.26
	2	1.00	5.46	14.39	40.07
	3	1.00	5.47	17.27	38.58
23	1	1.00	1.00	4.70	9.86
	2	1.00	1.00	11.33	7.06
	3	1.00	1.00	5.34	11.08

APPENDIX I WORKSHEET DATA – continued –

Table 10 Calculation of growth rate and yield

Average conc. [redacted] (mg/l)	Vessel number	Growth rate (μ) 0-72 h	Yield ($x10^4$ cells/ml) 0-72 h	Growth rate red. (%) 0-72 h	Yield inhib. (%) 0-72 h
control	1	0.07988	313.52		
	2	0.08279	386.83		
	3	0.07828	279.45		
	4	0.07951	305.31		
	5	0.08099	339.77		
	6	0.08100	339.98		
	mean	0.08041	327.48		
	CV	2%			
< LOD	1	0.07855	284.93	2	13
	2	0.08018	320.43	0	2
	3	0.07988	313.54	1	4
< LOD	1	0.07776	269.00	3	18
	2	0.08517	459.50	-6	-40
	3	0.06938	146.75	14	55
0.21	1	0.07833	280.38	3	14
	2	0.07771	268.13	3	18
	3	0.07729	260.09	4	21
0.86	1	0.07292	189.59	9	42
	2	0.07617	239.91	5	27
	3	0.07599	236.78	5	28
5.4	1	0.05355	46.26	33	86
	2	0.05126	39.07	36	88
	3	0.05073	37.58	37	89
23	1	0.03178	8.86	60	97
	2	0.02715	6.06	66	98
	3	0.03340	10.08	58	97

APPENDIX I WORKSHEET DATA – continued –

Table 11 Calculation of growth rate (section-by-section)

Average conc. [redacted] (mg/l)	Vessel number	Growth rate (μ)			Growth rate reduction (%)		
		0-24 h	24-48 h	48-72 h	0-24 h	24-48 h	48-72 h
control	1	0.08793	0.08050	0.07120			
	2	0.06941	0.08857	0.09038			
	3	0.06673	0.10613	0.06199			
	4	0.06901	0.11356	0.05596			
	5	0.07622	0.10877	0.05797			
	6	0.06829	0.11831	0.05639			
	mean	0.07293	0.10264	0.06565			
	CV	11%	14%	20%			
	The mean CV for section-by-section specific growth rate was: 25%						
< LOD	1	0.06797	0.09209	0.07560	7	10	-15
	2	0.07156	0.09445	0.07453	2	8	-14
	3	0.07541	0.09348	0.07074	-3	9	-8
< LOD	1	0.06837	0.09188	0.07301	6	10	-11
	2	0.07837	0.09341	0.08372	-7	9	-28
	3	0.05869	0.09717	0.05229	20	5	20
0.21	1	0.07754	0.07487	0.08258	-6	27	-26
	2	0.07396	0.08431	0.07487	-1	18	-14
	3	0.07111	0.08300	0.07776	3	19	-18
0.86	1	0.07252	0.07861	0.06763	1	23	-3
	2	0.07975	0.07884	0.06993	-9	23	-7
	3	0.08283	0.08083	0.06432	-14	21	2
5.4	1	0.07103	0.04328	0.04634	3	58	29
	2	0.07073	0.04038	0.04267	3	61	35
	3	0.07080	0.04790	0.03349	3	53	49
23	1	0.00000	0.06448	0.03087	100	37	53
	2	0.00000	0.10114	0.00000	100	1	100
	3	0.00000	0.06980	0.03041	100	32	54

APPENDIX II STATISTICS: GROWTH RATE (0-72 HOURS)

Chi-Square Test for Normality

Actual and Expected Frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.6080	5.8080	9.1680	5.8080	1.6080
OBSERVED	0	8	8	7	1

Chi-Square = 3.0586 (p-value = 0.5481)

Critical Chi-Square = 13.277 (alpha = 0.01 , df = 4)
= 9.488 (alpha = 0.05 , df = 4)

Data PASS normality test (alpha = 0.01). Continue analysis.

Levene's Test for Homogeneity of Variance

ANOVA Table

SOURCE	DF	SS	MS	F
Between	6	0.0001	0.0000	2.2186
Within (Error)	17	0.0001	0.0000	
Total	23	0.0001		

(p-value = 0.0918)

Critical F = 4.1015 (alpha = 0.01, df = 6,17)
= 2.6987 (alpha = 0.05, df = 6,17)

Since F < Critical F FAIL TO REJECT Ho: All equal (alpha = 0.01)

ANOVA Table

SOURCE	DF	SS	MS	F
Between	6	0.0069	0.0012	114.8334
Within (Error)	17	0.0002	0.0000	
Total	23	0.0071		

(p-value = 0.0000)

Critical F = 4.1015 (alpha = 0.01, df = 6,17)
= 2.6987 (alpha = 0.05, df = 6,17)

Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

APPENDIX II STATISTICS: GROWTH RATE (0-72 HOURS) – continued –

William's Test - TABLE 1 OF 2 Ho: Control<Treatment

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	control	6	0.0804	0.0804	0.0804
2	< LOD	3	0.0795	0.0795	0.0795
3	< LOD	3	0.0774	0.0774	0.0776
4	0.21	3	0.0778	0.0778	0.0776
5	0.86	3	0.0750	0.0750	0.0750
6	5.4	3	0.0518	0.0518	0.0518
7	23	3	0.0308	0.0308	0.0308

William's Test - TABLE 2 OF 2 Ho: Control<Treatment

IDENTIFICATION	COMPARED MEANS	CALC. WILLIAMS	SIG 0.05	TABLE WILLIAMS	DEGREES OF FREEDOM USED
control	0.0804				
< LOD	0.0795	0.3886		1.7400	k= 1, v=17
< LOD	0.0776	1.2491		1.8200	k= 2, v=17
0.21	0.0776	1.2491		1.8500	k= 3, v=17
0.86	0.0750	2.3993	*	1.8700	k= 4, v=17
5.4	0.0518	12.7337	*	1.8700	k= 5, v=17
23	0.0308	22.1274	*	1.8800	k= 6, v=17

s = 0.0032

APPENDIX III STATISTICS: YIELD (0-72 HOURS)

Chi-Square Test for Normality

Actual and Expected Frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.6080	5.8080	9.1680	5.8080	1.6080
OBSERVED	0	8	8	7	1

Chi-Square = 3.0586 (p-value = 0.5481)

Critical Chi-Square = 13.277 (alpha = 0.01 , df = 4)
= 9.488 (alpha = 0.05 , df = 4)

Data PASS normality test (alpha = 0.01). Continue analysis.

Levene's Test for Homogeneity of Variance

ANOVA Table

SOURCE	DF	SS	MS	F
Between	6	23763.8481	3960.6414	2.9679
Within (Error)	17	22686.2474	1334.4851	
Total	23	46450.0955		

(p-value = 0.0360)

Critical F = 4.1015 (alpha = 0.01, df = 6,17)
= 2.6987 (alpha = 0.05, df = 6,17)

Since F < Critical F FAIL TO REJECT Ho: All equal (alpha = 0.01)

ANOVA Table

SOURCE	DF	SS	MS	F
Between	6	344600.2146	57433.3691	16.5306
Within (Error)	17	59064.3413	3474.3730	
Total	23	403664.5559		

(p-value = 0.0000)

Critical F = 4.1015 (alpha = 0.01, df = 6,17)
= 2.6987 (alpha = 0.05, df = 6,17)

Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

APPENDIX III STATISTICS: YIELD (0-72 HOURS) – continued –

William's Test - TABLE 1 OF 2 Ho: Control<Treatment

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	control	6	327.4767	327.4767	327.4767
2	< LOD	3	306.3000	306.3000	306.3000
3	< LOD	3	291.7500	291.7500	291.7500
4	0.21	3	269.5333	269.5333	269.5333
5	0.86	3	222.0933	222.0933	222.0933
6	5.4	3	40.9700	40.9700	40.9700
7	23	3	8.3333	8.3333	8.3333

William's Test - TABLE 2 OF 2 Ho: Control<Treatment

IDENTIFICATION	COMPARED MEANS	CALC. WILLIAMS	SIG 0.05	TABLE WILLIAMS	DEGREES OF FREEDOM USED
control	327.4767				
< LOD	306.3000	0.5081		1.7400	k= 1, v=17
< LOD	291.7500	0.8572		1.8200	k= 2, v=17
0.21	269.5333	1.3902		1.8500	k= 3, v=17
0.86	222.0933	2.5284	*	1.8700	k= 4, v=17
5.4	40.9700	6.8740	*	1.8700	k= 5, v=17
23	8.3333	7.6571	*	1.8800	k= 6, v=17

s = 58.9438

APPENDIX IV EC-VALUES

Table 12 EC-values for growth rate reduction

Concentration (mg/l)	X Log conc. (mg/l)	Y Reduction (%)
< LOD	*	2.3
< LOD	*	0.3
< LOD	*	0.7
< LOD	*	3.3
< LOD	*	-5.9
< LOD	*	13.7
0.21	*	2.6
0.21	*	3.4
0.21	*	3.9
0.86	-0.066	9.3
0.86	-0.066	5.3
0.86	-0.066	5.5
5.4	0.732	33.4
5.4	0.732	36.3
5.4	0.732	36.9
23	1.362	60.5
23	1.362	66.2
23	1.362	58.5

* Not included in the EC calculations

Slope:	38.4521
Intercept:	8.6419
Multiple R:	0.9937
n = number of observations:	9

Regression line: Y= 38.45X + 8.64

Prediction of X values based on known Y values

Known Y Reduction (%)	10^{Xreg} (mg/l)	$10^{X95\%-}$ (mg/l)	$10^{X95\%+}$ (mg/l)
10	1.08	0.69	1.71
20	1.97	1.28	3.05
50	11.90	7.68	18.43

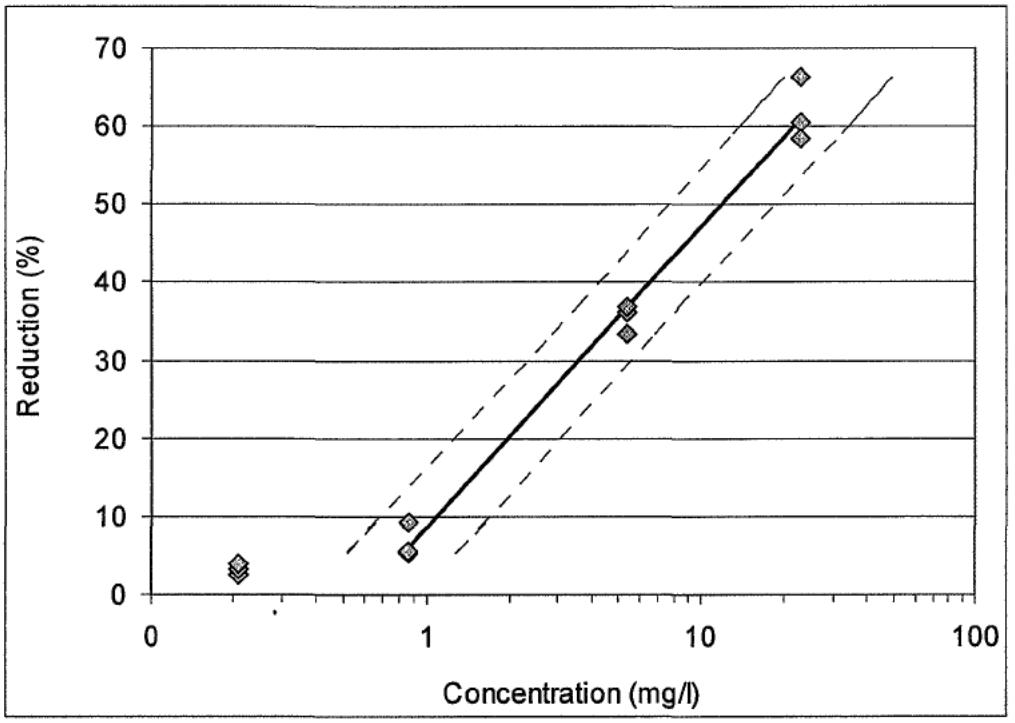

Figure 2 **Percentage reduction of growth rate as function of the log concentration (mg/l) of [redacted] Dashed curves represent the 95% confidence limits.**

APPENDIX IV EC-VALUES – continued –

Table 13 EC-values for yield inhibition

Concentration (mg/l)	X Log conc. (mg/l)	Y Inhibition (%)
< LOD	*	13.0
< LOD	*	2.2
< LOD	*	4.3
< LOD	*	17.9
< LOD	*	-40.3
< LOD	*	55.2
0.21	-0.678	14.4
0.21	-0.678	18.1
0.21	-0.678	20.6
0.86	-0.066	42.1
0.86	-0.066	26.7
0.86	-0.066	27.7
5.4	0.732	85.9
5.4	0.732	88.1
5.4	0.732	88.5
23	*	97.3
23	*	98.1
23	*	96.9

* Not included in the EC calculations

Slope:	50.4727
Intercept:	45.9710
Multiple R:	0.9589
n = number of observations:	9

Regression line: Y= 50.47X + 45.97

Prediction of X values based on known Y values

Known Y Inhibition (%)	10^{Xreg} (mg/l)	$10^{X95\%-}$ (mg/l)	$10^{X95\%+}$ (mg/l)
10	0.19	0.06	0.64
20	0.31	0.10	0.97
50	1.20	0.39	3.66

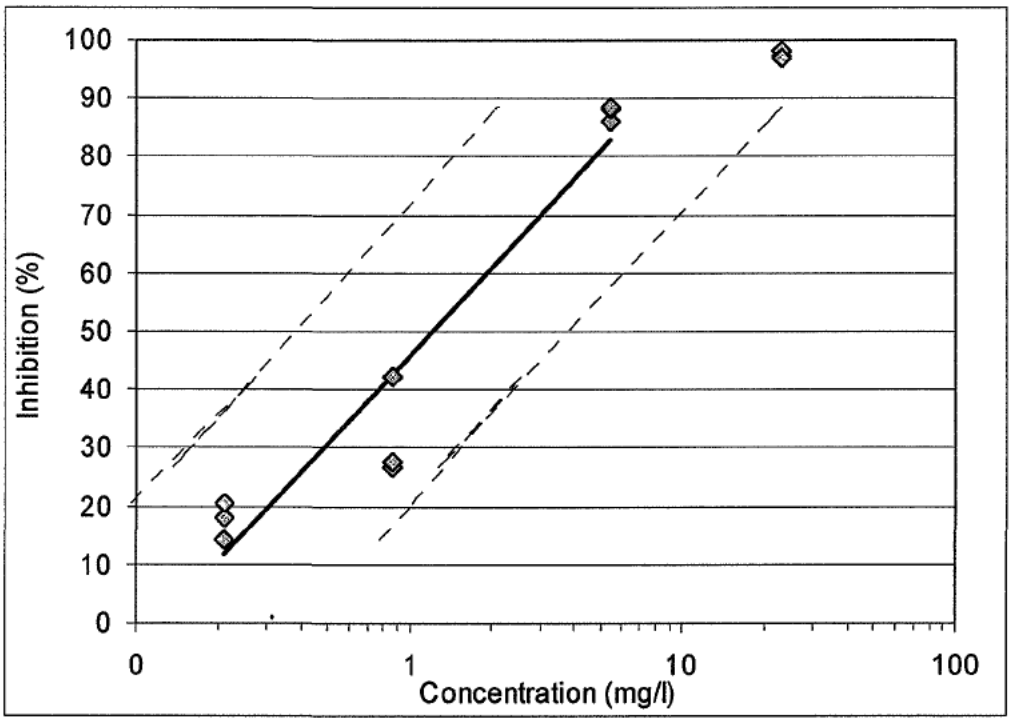

Figure 3 **Percentage inhibition of yield as function of the log concentration (mg/l) of [redacted] Dashed curves represent the 95% confidence limits.**

APPENDIX V REFERENCE TEST

Pseudokirchneriella subcapitata, strain: NIVA CHL-1. Fresh water algal growth inhibition test with potassium dichromate (NOTOX Project 488286).

Start of first exposure: 14 April 2008
Completion last exposure: 17 April 2008

The study procedures described in this report were based on the EEC Directive 92/69, Publication No. L383 Part C-3 adopted December, 1992; OECD guideline No. 201, Adopted March 23, 2006; and ISO Standard 8692, Second edition, 01 October 2004.

This reference test was carried out to check the sensitivity of the test system used by NOTOX to Potassium dichromate (Merck, Art. 4864, Batch K34869764 607).

Algae were exposed for a period of 72 hours to $K_2Cr_2O_7$ (Potassium dichromate) concentrations of 0.18, 0.32, 0.56, 1.0, 1.8 and 3.2 mg/l and to a control. The initial cell density was 1.0×10^4 cells/ml.

Results:

Overview of % reduction of growth rate and % inhibition of yield in the reference test:

Nominal conc. $K_2Cr_2O_7$ (mg/l)	Mean growth rate		Yield (0-72 h)	
	μ (0-72 h)	Reduction (%)	$x10^4$ cells/ml	Inhibition (%)
control	0.07433		210.81	
0.18	0.07515	-1.1	223.61	-6.1
0.32	0.07510	-1.0	222.30	-5.5
0.56	0.07200	3.1	177.54	15.8
1.0	0.05176	30.4	40.64	80.7
1.8	0.03235	56.5	9.27	95.6
3.2	0.02613	64.8	5.57	97.4

Under the conditions of the reference study with *Pseudokirchneriella subcapitata*, potassium dichromate reduced growth rate of this fresh water algae species at nominal concentrations of 1.0 mg/l and higher.

The EC_{50} for growth rate reduction (E_RC_{50}: 0-72h) was 1.8 mg/l with a 95% confidence interval ranging from 1.3 to 2.6 mg/l. The historical ranges for growth rate reduction lie between 0.82 and 2.3 mg/l. Hence, the E_RC_{50}: 0-72h for the present batch corresponds with this range.

The EC_{50} for yield inhibition (E_YC_{50}: 0-72h) was 0.79 mg/l with a 95% confidence interval ranging from 0.51 to 1.2 mg/l. Historical ranges are not yet available.

The protocol, raw data and report of this study are kept in the NOTOX archives. The test described above was performed under GLP conditions with a QA-check.

APPENDIX VI

FRESH WATER ALGAL GROWTH INHIBITION TEST WITH [redacted]
DETERMINATION OF THE CONCENTRATIONS

Author

[redacted]

Study completion date

11 August 2008

Laboratory Project Identification

NOTOX [redacted]
NOTOX Substance [redacted]

1. CONTENTS

2. REPORT APPROVAL

PRINCIPAL SCIENTIST: [redacted]
(Analytical Chemistry)

[redacted]

Date: 11 August 2008

3. INTRODUCTION

3.1. Preface

Study plan	Start	:	13 May 2008
(analytical study)	Completion	:	14 May 2008

3.2. Aim of the study

The purpose of the analytical study was to determine the actual concentrations in samples taken from the test solutions used during the ecotoxicity test.

4. MATERIALS AND METHODS

4.1. Reagents

Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA)
Methanol	HiPerSolv Chromanorm, VWR International, Leuven, Belgium
Tetrahydrofuran	Merck, Darmstadt, Germany
Trifluoroacetic acid	for synthesis, Merck
M2-medium	see main report

All reagents were of analytical grade, unless specified otherwise.

4.2. Samples

The samples were stored in the freezer till the day of analysis. Storage stability of samples under these conditions was determined in NOTOX [redacted] On the day of analysis, the frozen samples were defrosted at room temperature. The test samples were diluted in a [redacted] with methanol, and analysed. If necessary, the samples were further diluted with [redacted] methanol/M2-medium to obtain concentrations within the calibration range.

4.3. Analytical method

4.3.1. Analytical conditions

Quantitative analysis was based on the analytical method validated for the test substance in NOTOX [redacted].

Analytical conditions:

Instrument	Alliance Separation Module 2695 (Waters, Milford, MA, USA)
Detector	Dual λ Absorbance Detector 2487 (Waters)
Column	[redacted] i.d. Symmetry Shield RP-18, [redacted] (Waters)
Column temperature	35°C
Injection volume	100 µl
Mobile phase	A – 0.02% trifluoroacetic acid in Milli-Q water B – 0.02% trifluoroacetic acid in tetrahydrofuran

Gradient

Time [minutes]	%A	%B
0	90	10
8	0	100
8.1	90	10
13	90	10

Flow 1.0 ml/min
UV detection [redacted]

4.3.2. Preparation of the calibration solutions

Stock- and spiking solutions
Stock solutions of the test substance were prepared in methanol at concentrations of [redacted]

Spiking solutions were made up from a stock solution and a dilution of this solution. The solvent of the spiking solutions was methanol.

Calibration solutions
Calibration solutions in the concentration range 0.04 – 9.95 mg/l were prepared from two stock solutions. The end solution of the calibration solutions was 50/50 (v/v) methanol/M2-medium.

Procedural recovery samples
2 ml blank medium was spiked with the test substance at a target concentration of [redacted] The accuracy samples were treated similarly as the test samples (see paragraph 4.2 'Samples').

4.3.3. Sample injections

Calibration solutions were injected in duplicate. Test samples and procedural recovery samples were analysed by single injection.

4.4. Electronic data capture

System control, data acquisition and data processing were performed using the following programme:
- Empower version 5.00 (Waters, Milford, MA, USA).

Temperature and/or relative humidity during sample storage and/or performance of the studies were monitored continuously using the following programme:
- REES Monitoring system version 1.5 (REES Scientific, Trenton, NJ, USA).

4.5. Formulas

Response (R)

Peak area test substance [units]

Calibration curve

$$R = a\,C_N + b$$

where:
C_N = nominal concentration [mg/l]
a = slope [units × l/mg]
b = intercept [units]

Analysed concentration (C_A)

$$C_A = \frac{(R - b)}{a} \times d \quad [mg/l]$$

where:
d = dilution factor

Recovery

$$\frac{C_A}{C_N} \times 100\%$$

Relative to initial concentration

$$\frac{C_A\,(t = x\ hours)}{C_A\,(t = 0\ hours)} \times 100\%$$

Expected concentration (C_{exp})

$$\frac{percentage\ of\ WSF}{100} \times C_{undil.}$$

$C_{undil.}$ = nominal concentration of undiluted water soluble fraction (WSF) at t=0 hours [mg/l]

Relative to expected concentration

$$\frac{C_A}{C_{exp}} \times 100\%$$

Limit of detection (LOD)

$$LOD = \frac{3\,N}{S} \times C_N$$

where:
N = noise height [units]
S = peak height [units]

5. RESULTS

5.1. Calibration curves

Calibration curves were constructed using eight concentrations. The data points of the 0.04 and 0.12 mg/l calibration solutions deviated more than 10% from the calculated line and were therefore excluded. The remaining six concentrations were in the range of [redacted]. For each concentration, two responses were used. Linear regression analysis was performed using the least squares method with a 1/concentration^2 weighting factor. The coefficient of correlation (r) was > 0.99.

Representative chromatograms of a test substance solution and blank solution are shown in Figure 1.

5.2. Samples

5.2.1. Procedural recovery samples

The results for the procedural recovery samples are given in Tables 1-3.

Procedural recovery samples based on [redacted]

[redacted] for the [redacted] This was considered acceptable since the coefficient of variation was 4.4 and 0.94% respectively. [redacted] concentrations in the test samples are considered accurate based on analysis of the [redacted]

Procedural recovery samples based on [redacted]

Mean recoveries of the procedural recovery samples analysed for [redacted] The coefficient of variation was 12% and 1.1% respectively. Concentrations measured in the test samples are expected to have a similar spread and are at lower concentration possibly overestimated. Therefore [redacted] concentrations in the test samples have to be considered indicative based on analysis of [redacted].

Procedural recovery samples based on [redacted]

Mean recoveries of the procedural recovery samples analysed for [redacted] were 109% and 98% respectively at 0.32 and 100 mg/l [redacted] The coefficient of variation was 17% and 1.2% respectively. Concentrations measured in the test samples are expected to have a similar spread. Therefore [redacted] concentrations in the test samples at lower concentrations have to be considered indicative based on analysis of [redacted]

5.2.2. Test samples

The results for the test samples are given in Tables 4, 5 and 6. The results based on analysis of [redacted] have to be considered indicative.

6. TABLES

Table 1 **Procedural recovery samples based [redacted]**

Date of preparation [dd-mm-yy]	Date of analysis [dd-mm-yy]	Target concentration [mg/l]	Nominal concentration [mg/l]	Analysed concentration [mg/l]	Recovery [%]	Mean recovery (coefficient of variation) [%]
13-05-08	13-05-08	[redacted]			120 113 115 108 108	113 (4.4)
13-05-08	13-05-08				102 103 102 103 101	102 (0.94)

[1] Obtained by extrapolation of the calibration curve. Result is indicative.

Table 2 **Procedural recovery samples based on [redacted]**

Date of preparation [dd-mm-yy]	Date of analysis [dd-mm-yy]	Target concentration [mg/l]	Nominal concentration [mg/l]	Analysed concentration [mg/l]	Recovery [%]	Mean recovery (coefficient of variation) [%]
13-05-08	13-05-08	[redacted]			126 114 129 129 95	125 (12)
13-05-08	13-05-08				97 98 97 99 98	98 (1.1)

[1] Obtained by extrapolation of the calibration curve. Result is indicative.

Table 3 Procedural recovery samples based [redacted]

Date of preparation [dd-mm-yy]	Date of analysis [dd-mm-yy]	Target concentration [mg/l]	Nominal concentration [mg/l]	Analysed concentration [mg/l]	Recovery [%]	Mean recovery (coefficient of variation) [%]
13-05-08	13-05-08	[redacted]			92 119 95 104 136	109 (17)
13-05-08	13-05-08				97 99 97 100 99	98 (1.2)

[1] Obtained by extrapolation of the calibration curve. Result is indicative.

Table 4 Concentrations of the test substance in test medium based on the [redacted] (final test)

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [1] [dd-mm-yy]	Concentration				
			Percentage of WSF [2] [%]	Expected [3] [mg/l]	Analysed [mg/l]	Relative to expected [%]	Relative to initial [%]
0	05-05-08	13-05-08	[redacted]			151	
						97	
						92	
						96	
						90	
						96	
24	06-05-08	13-05-08				136	90
						105	108
						93	101
						90	94
						91	101
						114	119
						107	107
72	08-05-08	13-05-08				131	87
						95	98
						153	167
						101	105
						165	183
						142	148
						107	107

1 Samples were stored in the freezer until the day of analysis.

2 Percentage of a water soluble fraction (WSF) prepared at a loading rate of [redacted]

3 Based on the concentration analysed in the 100% test solution without algae.

4 Obtained by extrapolation of the calibration curve. Result is indicative.

5 Without algae.

Table 5 **Concentrations of the test substance in test medium based on [redacted] (final test)**

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [1] [dd-mm-yy]	Concentration				
			Percentage of WSF [2] [%]	Expected [3] [mg/l]	Analysed [mg/l]	Relative to expected [%]	Relative to initial [%]
0	05-05-08	13-05-08	[redacted]			143	
						100	
						92	
						93	
						91	
						95	
24	06-05-08	13-05-08					
						87	87
						84	91
						94	101
						96	105
						120	126
						106	106
72	08-05-08	13-05-08					
							0
						84	84
						129	140
						94	101
						143	156
						128	134
						105	105

1 Samples were stored in the freezer until the day of analysis.

2 Percentage of a water soluble fraction (WSF) prepared at a loading rate of [redacted]

3 Based on the average concentration analysed in the 100% test solution without algae.

4 Obtained by extrapolation of the calibration curve. Result is indicative.

5 Without algae.

LOD The limit of detection was determined to be [redacted] taking a dilution factor of two into account.

Table 6 **Concentrations of the test substance in test medium based on [redacted] (final test)**

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [1] [dd-mm-yy]	Concentration				
			Percentage of WSF [2] [%]	Expected [3] [mg/l]	Analysed [mg/l]	Relative to expected [%]	Relative to initial [%]
0	05-05-08	13-05-08	[redacted]				
						97	
						84	
						81	
						96	
24	06-05-08	13-05-08					
						22	26
						54	66
						85	89
						76	76
72	08-05-08	13-05-08					
						35	41
						74	91
						92	96
						74	74

1 Samples were stored in the freezer until the day of analysis.

2 Percentage of a water soluble fraction (WSF) prepared at a loading rate of [redacted]

3 Based on the average concentration analysed in the 100% test solution without algae.

4 Without algae.

LOD The limit of detection was determined to be [redacted] taking a dilution factor of two into account.

7. FIGURES

Figure 1 **Chromatograms of a [redacted] test substance solution [top; [redacted] and corresponding blank [bottom; res. id.1566]. UV detection at [redacted]**

Figure 2 **Chromatograms of a [redacted] test substance solution [top; [redacted] and corresponding blank [bottom; res. id. 1707]. UV detection [redacted]**